Published in final edited form as:
Wiley Interdiscip Rev Syst Biol Med. 2009 September 1; 1(2): 237–246. doi:10.1002/wsbm.24.

Experimentally Based Sea Urchin Gene Regulatory Network and the Causal Explanation of Developmental Phenomenology

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Abstract

Gene regulatory networks for development underlie cell fate specification and differentiation. Network topology, logic and dynamics can be obtained by thorough experimental analysis. Our understanding of the gene regulatory network controlling endomesoderm specification in the sea urchin embryo has attained an advanced level such that it explains developmental phenomenology. Here we review how the network explains the mechanisms utilized in development to control the formation of dynamic expression patterns of transcription factors and signaling molecules. The network represents the genomic program controlling timely activation of specification and differentiation genes in the correct embryonic lineages. It can also be used to study evolution of body plans. We demonstrate how comparing the sea urchin gene regulatory network to that of the sea star and to that of later developmental stages in the sea urchin, reveals mechanisms underlying the origin of evolutionary novelty. The experimentally based gene regulatory network for endomesoderm specification in the sea urchin embryo provides unique insights into the system level properties of cell fate specification and its evolution.

Keywords

gene regulation in development; evolution; systems level properties

Introduction

Networks of regulatory genes control cell fate specification and differentiation of developing embryos [1]. The network linkages are encoded in the genomic sequences regulating gene expression. These regulatory sequences are termed *cis*-regulatory modules; they contain clusters of binding sites for transcription factors which function to activate or repress the expression of the genes they control. The functions the *cis*-regulatory element executes on its inputs can be described in terms of the basic logic functions AND, Additive OR and NOT [2–4]. The regulatory genes that are part of a network, the linkages between them, the logic their *cis*-regulatory elements execute on their inputs, can be determined experimentally, following the analysis presented in Fig. 1 [5,6]. This analysis was conducted for the gene regulatory network controlling the endomesoderm specification of sea urchin embryo. The network can now be used to explain observed developmental phenomenology and to gain insights into the general design principles of gene regulatory networks for development [7–11].

There are typical developmental tasks required for embryogenesis to progress. Among these are pattern formation in space and time, timely activation of specification states, and activation of the correct sets of differentiation genes in the right cell lineage. We are now able to specify how these tasks are regulated by the circuits of the gene regulatory network. The instructions for creating dynamic spatial patterns are encoded in the *cis*-regulatory

elements of signaling molecules and transcription factors [8,10,11]. The timely activation of regulatory genes is encoded in their *cis*-regulatory elements often arranged in the form of a cascade of feed-forward loops [7,12]. Other typical network motifs such as positive and negative feedback loops [10,11,13,14] are utilized in the gene regulatory network to run the developmental process correctly and reliably.

Another fundamental problem that can be addressed by a detailed model of gene regulatory networks is their evolution. Comparing the topology of gene regulatory networks of two or more related species teaches us about the evolutionary mechanisms underlying change, as well as conservation, of embryo body plans. Studying the changes in the *cis*-regulatory elements of regulatory genes can illuminate the mechanisms of network topology modification. Thus we have found that gene regulatory network governing endomesoderm specification in the sea star produces an instructive comparison with that of the sea urchin [15–17].

In this paper we demonstrate the explanatory power of the gene regulatory network that controls the sea urchin endomesoderm specification. We present regulatory circuits that underlie fundamental developmental processes, such as dynamic patterning of gene expression, cell fate specification, and the order of genes activation in the developmental time. These examples were acquired through the experimental analysis described in the flow chart in Fig. 1 (see refs. [5,6], for detailed protocols). From this model we can now learn about the general principles of the regulation and evolution of the specification process.

Gene regulatory network circuit controlling a dynamic spatio-temporal pattern of Wnt8 and Delta pathways

The Wnt and Notch signaling pathways are commonly used in cell fate specification. Periodic oscillation of these two pathways lie beneath the rhythmic production of the vertebral precursors, the somites, from the presomitic mesoderm [18,19]. Both pathways also participate in T-cell specification [20].Different components of the pathways were shown to interact with each other [21]. However, a direct regulatory link connecting the two pathways and enabling a controlled progressive activation and segregation of these signaling pathways has not previously been shown. Very recently, the explicit *cis*-regulatory links between them were found in the sea urchin gene regulatory network, where the two pathways play key roles in the endomesoderm specification process [10].

This dynamic regulatory circuit is active in the presumptive mesoderm and endoderm of the sea urchin embryo. These territories arise as follows. The fourth cleavage is unequal, and results in small and large tiers of cells, the micromeres and the macromeres, respectively. At fifth cleavage the micromeres divide into small and large micromeres (Fig. 2A). The large micromeres marked in red in Fig. 2A, emit signals that are required for specification of the vegetal plate. The descendents of the large micromeres ingress into the blastocoel at about 20 hours post-fertilization (hpf) and later fuse to form the skeleton (Fig. 2A). (From now on, the hours mentioned are for the sea urchin *S. purpuratus*, grown at 15°C.) These cells are the skeletogenic mesenchyme cells (SMs). They are the first to ingress into the blastocoel and assume mesenchymal form. The macromere descendants form the remaining mesoderm and the endoderm. The tier of cells adjacent to the SM descendants is destined to give rise to mesoderm and from it derive several distinct mesodermal cell lineages, e.g., pigment cells and blastocoelar cells. These cells are termed non-skeletogenic mesoderm cells (NSM) and are marked in purple in Fig. 2A. The next tiers of cells, marked in blue in Fig. 2A give rise to the endoderm. Gastrulation begins at about 30 hpf with the invagination of endodermal cells which will form the embryonic gut.

The transcriptional activation of the gene encoding the Wnt8 ligand requires two inputs, TCF-βcatenin and Blimp1 [22]. Due to maternal determinants localized before fertilization, βcatenin is nuclearized only in the vegetal most cells of the embryo [9,23,24]. The Blimp1 transcription factor is maternal, i.e., it is present in the egg, and its mRNA is in all the cells of the embryo. However, zygotic expression of the *blimp1* gene, that is, its post-fertilization expression, is activated by TCF-βcatenin and Otx [11]. Therefore, early in development both *blimp1* and *wnt8* are activated in the micromeres, where all their inputs are present, Fig. 2B. The reception of the Wnt8 signal in the next tier of cells leads to further nuclearization of βcatenin and to the activation of *wnt8* and *blimp1* there. However, once Blimp1 reaches high enough levels it shuts down its own expression, and as a result, *wnt*8 transcription is also shut down [11]. These two feedback loops, one positive (Wnt8-βcatenin) and one negative (Blimp1 auto-repression) induce a dynamic expression pattern expanding from the vegetal pole of the embryo toward the animal pole, because the Wnt8 ligand diffuses outward to adjacent concentric tiers of cells even while transcription of *wnt8* and *blimp1* genes is extinguished in their earlier expression domains [11] (Fig. 1B).

The gene encoding the Delta ligand is repressed by the transcriptional repressor HesC and is activated by the then ubiquitous transcription factor Runx [10,25]. The *hesc* gene is initially expressed zygotically all over the embryo but at about 6 hpf the repressor *pmar1* turns on in the micromeres and shuts down *hesc* [26] (Fig. 2B). As a result *delta* is activated in these cells at about 8 hpf, Fig. 2B. At this time in development, the two ligands, *wnt8* and *delta* are expressed in the same cells (Fig. 2B). However, 3 hours afterwards, *wnt8* expression expands to the next tier of cells and the *blimp1* gene starts repressing both itself and the *hesc* gene as well in the micromeres [10] (Fig. 2B). The silencing of *hesc* by Blimp1 enables the continuation of *delta* expression in the micromere descendents even after *pmar1* is turned off, at 15 hpf. Thus at 15 hpf *delta* is still being expressed in the micromere descendents, while *blimp1* and *wnt8* have been shutoff there, but are now activated in the next tiers of cells, the NSM precursors (Fig. 2B). Blimp1 silencing of *hesc* and itself therefore leads to the spatial segregation of the two pathways.

As the Wnt8-Blimp1 circuit advances to the next tier of cells, the NSM, so does *hesc* silencing. At about 20 hpf, the micromere descendents ingress into the blastocoel, and *delta* is turned off in this lineage. Blimp1 clears itself, *wnt8* and *hesc* from the NSMs. This leads to the activation of *delta* in these cells. At this time *wnt8* is expressed in the endodermal cells and *delta* is expressed in the NSM cells. As shown in Fig. 2C expression of the genes encoding these two ligands is segregated by the regulatory network subcircuit that connects the two pathways.

Two other interesting links in this circuit are Blimp1 silencing of the *notch* gene and Notch activation of the *hesc* gene (Fig. 1C [10]). These two links contribute to the characteristic inhibitory relation observed in the Delta-Notch pathway: *notch* is repressed by Blimp1, the same factor that enables the expression of *delta*. Therefore in the cells expressing *delta*, *notch* is repressed. On the other hand, in the cells that receive the Delta signal the Notch receptor is cleaved which results in activation of the repressor of *delta*, *hesc*. In these cells the *notch* gene is active and *delta* expression is repressed.

This network circuit demonstrates how the dynamic spatiotemporal expression pattern of transcription factors and signaling molecules is encoded in their static *cis*-regulatory elements. The *cis*-regulatory linkages of this subcircuit define the program that is executed through development, and in this case they lead to sequential activation and exclusive segregation of *wnt8* and *delta* expression pattern in the endomesodermal cells.

The skeletogenic lineage gene regulatory network

The micromere descendents, and only them, give rise to the skeletogenic mesenchyme (SM) lineage. The gene regulatory network underlying the specification of this lineage includes about 30 transcription factors, signaling molecules and differentiation genes [7]. In this section we analyze the essential circuits of the network, the program that includes a cascade of regulatory events extending from the initiation of the micromere specification state to the activation of SM differentiation genes.

The HesC repressor that was mentioned in the previous section represses a set of genes encoding transcription factors. When Pmar1 removes HesC repression in the micromeres, these genes are activated and the specification program of the SM lineage sets off [7]. In Fig. 3A we present a diagram of the double negative gate, showing the linkages which cause activation of two of the key micromere specification genes, *ets1* and *alx1* and two of their downstream genes. Figure 3B shows the spatial expression patterns of *pmar1, hesc, ets1* and *alx1* at late 5th cleavage, at about 8 hpf in the sea urchin *S. purpuratus*. The Ets1 factor is initially maternal. The zygotic expression of the *ets1* gene is repressed by HesC, and therefore it is activated only in the micromeres. At 8 hpf there are still remains of the maternal Ets1 in other domains of the embryo, but in the micromeres Ets1 transcript levels are significantly higher than elsewhere (Fig. 3B). The *alx1* gene is repressed by HesC and activated by Ets1. The global repression by HesC prevents *alx1* activation outside the micromere lineage, even though its activator Ets1 is present there (Fig. 3B).

Later in development both Ets1 and Alx1 are localized in the micromere descendants and so are the products of their downstream genes, the transcription factor Dri and the differentiation gene *cyclophilin* (Fig. 3C). *cyclophilin* is among the differentiation genes activated in the skeletogenic territory as a terminal function of the endomesodermal gene regulatory network. The genes *ets1, alx1, dri* and *cyclophilin* form a cascade of feedforward loops: Ets1 activates *alx1*, Ets1 and Alx1 together activate *dri* and then Ets1 and Dri activate *cyclophilin* (Fig. 1A).

In Fig. 3D we present the dynamic expression levels of the mRNA of these genes, measured by quantitative PCR (QPCR). *pmar1* is on at about 5 hpf, represses *hesc* in the micromere and that permits the activation of *alx1* at about 7 hpf. The structure of this subcircuit, the double negative logic and the maternal presence of the activator Ets1 allows for a fast turn on of the *alx1* gene. The size of *alx1* primary transcript is about 37 kb and it takes more than an hour for the RNA polymerase to transcribe this length at 15°C [27]. Adding to that the time required for mRNA transport from the nucleus, translation, and protein transport back to the nucleus, we obtain about 3–4 hours of approximate delay in the activation of its downstream genes. Indeed, *dri* is activated at about 11 hpf (Fig. 3D). The *cyclophilin* gene turns on at about the same time as *dri*. The *cis*-regulatory element of the *cyclophilin* gene was identified, and it contains functional binding sites for Ets1 and Dri [12]. The fact that both *dri* and *cyclophilin* turn on at the same time indicates that the input of Dri into the *cyclophilin cis*-regulatory module is additive to that of Ets1. That raises the question why *cyclophilin* is not on earlier and broader, since Ets1 is maternal. This point requires further experimental analysis.

This example of gene regulatory circuitry demonstrates how the network topology controls the localized spatial expression of the genes and their order of activation. The duration of a transcriptional step like the absolute level of transcripts depends also on the mRNA and protein initiation and turnover rates, and probably on the size of the primary transcript [27]. However, the spatial expression patterns and the order of events in the developmental time

depend mostly on the gene regulatory network wiring encoded in the *cis*-regulatory elements of the network genes.

Evolution of gene regulatory networks

When we analyze certain network architecture and try to understand why this specific architecture was selected, we have to consider the evolutionary constraints on the system. What was the basal network structure? How did novelties in network topology come about? Why are certain circuits more conserved then others? These are some of the fundamental questions we need to consider in the evolution of gene regulatory networks. Comparative studies of the gene regulatory network governing endomesoderm specification in a sea star were recently published [15–17]. The sea star and the sea urchin shared a common ancestor about 500 million years ago. Comparison between the gene regulatory networks of the sea urchin and the sea star reveals the regulatory network circuits underlying both conservation and changes in the body plans of these embryos.

There are two apparent novelties of the sea urchin embryo. One is the precociously specified skeletogenic micromere lineage discussed in the previous section, and the other is the early mesodermal specification of the pigment cell lineage. We can conclude that the sea star represents the ancestral situation in these respects, since the sister group of the sea urchins, the sea cucumbers, lacks both, as do the other echinoderm classes. In Fig. 4 we present a 72 hpf sea urchin larva possessing skeleton and pigment cells (Fig. 4A) and a 70 hpf sea star larva where these body parts are absent (Fig. 4B). Both animals make gut (endoderm) and blastocoelar cells (mesoderm). Key regulatory circuits governing endomesoderm specification in the sea urchin embryo and in the sea star embryo are shown in Figs. 4C, D. Below we identify the circuits controlling the specification of the two novel lineages, the skeleton and the pigment cells, and the two ancient ones, the blastocoelar cells and the endoderm.

The skeletogenic lineage circuit is missing in the sea star as expected. Both sea urchin and sea star are indirect developers and in both organisms the adult form generates extensive skeletal structures as do all echinoderms [28]. Similar biomineralization genes are expressed both in the adult skeleton and in the sea urchin embryonic one. Was the adult skeletogenesis program inserted into the embryonic micromere lineage? If so, what network linkages were added to enable that? In order to approach this question the expression of 24 genes of the micromere skeletogenic network were studied in the juvenile skeletonization centers of advanced larvae [28]. This study revealed that most of the network genes including almost all the transcription factors, biomineralization, and other differentiation genes, are expressed in the skeletonization centers of the juvenile sea urchin. More than that, the expression of eight key skeletonic regulatory genes and one downstream gene was studied in the sea star juvenile and the results were identical to those in sea urchin. This implies that the embryonic endomesodermal network was changed in the sea urchin so that it activates some of the juvenile skeletogenic program.

It would be interesting to try and track the changes in the network that lead to the activation of the skeletogenic program in the embryo. Many regulatory genes that are active in the embryonic micromere skeletogenic lineage are also active in the sea urchin NSMs (e.g., *ets1*), as well as in the sea star mesoderm (e.g., *ets1* and *tbr*). Their activity cannot explain the activation of the biomioneralization genes that are skeletogenesis specific. Two micromere specific genes are *pmar1* and *alx1*. These two are not expressed in the sea urchin NSM nor in the sea star endomesoderm. Apparently, the activation of *pmar1* early in the development of the micromeres, and its repression of *hesc*, is one part of the skeletogenic lineage insertion mechanism. Another part has to be the activation of *alx1* in the micromeres

early in development. *alx1* is a member of the Cart1/Alx3/Alx4 subfamily of Paired-class homeodomain proteins that were found to function in skeletogenesis in vertebrates [29,30]. However this is not all there is to it, as the obvious experiment, forcing *alx1* expression in the sea star embryo NSM, fails to induce embryonic skeletogenic expression in NSM cells (unpublished data).

As we explained above, there are two phases of Delta signaling in sea urchin, one from the micromeres to the NSM, and one from the NSM to the endoderm. The early phase is enabled due to the Pmar1 repression of *hesc*, which is a sea urchin novelty. In sea star Pmar1 is absent, so only the later phase of Delta signaling occurs, and the reception of the Delta signal is essential to endoderm specification [15]. Downstream of the early Delta signal in the sea urchin we can identify a sea urchin specific target, the *gcm* gene (Fig. 4C). The transcription factor Gcm is a key regulator of pigment cell specification [31]. When either the Delta-Notch pathway or *gcm* expression are perturbed in sea urchin embryos the pigment program is not turned onand the embryo lacks pigment cells [31]. The *gcm* gene is not expressed at all in the endomesoderm of the sea star. The early onset of the Delta signal by Pmar1 repression of HesC and the insertion of *gcm* as a target to this signal, enabled the specification of a new embryonic lineage, the pigment cell lineage, in the sea urchin. Whether *gcm* is utilized in the production of adult pigment cells in either or both of these animals is unknown.

Both the sea star and the sea urchin make a gut, approximately at the same developmental time. In agreement with that, the gene regulatory circuit controlling endoderm specification is highly conserved between the two organisms (Figs. 4C, D) [17]. The five transcription factors, Blimp1, Otxβ, Gatae, FoxA and Brachyury are essential for gut formation in both animals and the network wiring between these genes is almost identical. One of the few differences is the Tbr input into *otx*β, which is required in sea star but altogether absent in the sea urchin. Comparison between the *cis*-regulatory module of the *otx*β gene in the sea urchin to that of the sea star reveals the genomic mechanism of this change (Fig. 4E) [16]. Both *cis*-regulatory modules contain binding sites for the conserved inputs, Otx, GataE and Blimp1, but only the sea star *cis*-regulatory module contains binding sites for Tbr. Apparently, the sea urchin *otx*β *cis*-regulatory element lost its Tbr sites, in consequence of the redeployment of the *tbr* gene to the skeletogenic GRN and its removal from the endodermal GRN. Again we know the polarity of this change, since even in the sea cucumber sister group of the sea urchins *tbr* is expressed in the endoderm [32].

Another identity between the sea urchin and the sea star is the blastocoelar cells present in both (Figs. 4A, B). These cells are part of the immune system of the sea urchin and their primary function is protection against invasive marine pathogens. The transcription factor GataC, homolog of Gata1/2/3, is expressed in these cells in both organisms (Figs. 4C, D), and is a key regulator of blastocoelar cell specification [33]. The Gata1/2/3 transcription factors are important regulators of hematopoiesis in all bilateria and they play an important role in the specification of immune cells.

The comparison between the gene regulatory networks of the sea urchin and the sea star provides for us unprecedented views of the mechanisms underlying evolutionary modifications of body plans, as well as of examples of extreme conservation of detailed subcircuit wiring.

Future directions

The detailed experimental study of the gene regulatory network governing endomesoderm specification has enabled the construction of a model sufficient to explain characteristic developmental processes [7,8,11]. The network illuminates the regulation of dynamic spatial

gene expression patterns [8,10,11], and the timely activation of specification and differentiation genes in the correct spatial locations [7]. Network analysis enables a mechanistic understanding of the processes of cell fate specification and spatial pattern formation. Near future challenges include extending the network forward to later stages of increased complexity; extending it to additional domains so that eventually the whole embryo is included; and using the sea urchin model system as a guide to how to extend network analysis to diverse other biological systems. Knowledge of network structure and function will make it possible to rewire network connections and reengineer embryo development. Understanding the regulation of differentiation genes will provide the connection between gene regulatory networks downstream differentiation functions. Other kinds of linkages into gene regulatory networks control the basic functions of morphogenesis. Furthermore, comparative analysis of gene regulatory networks is a powerful lever that reveals the processes of evolution in unprecedented depth [15,17,28]. We will be able to follow evolutionary changes from the root changes in genomic sequence, to the effects on network wiring, to alterations in developmental function.

Acknowledgments

The authors thank Joel Smith for critical review of the manuscript and insightful comments. Research was supported by NIH grant GM-61005. Smadar Ben-Tabou de-Leon was supported by the Human Frontiers Science Program Organization.

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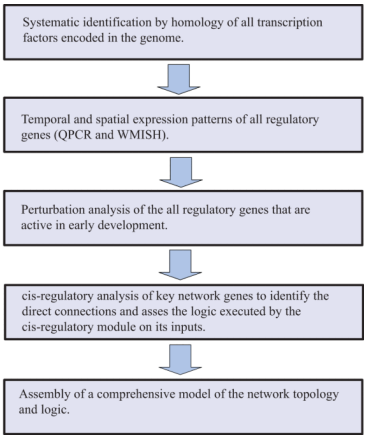

Figure 1.

Flow chart of the experimental analysis that was conducted in order to construct the gene regulatory network controlling endomesoderm specification in sea urchin embryo. The transcription factors of the sea urchin were identified computationally using the sea urchin genome [34–39]. The expression patterns and time courses of all the identified transcription factors were measured by WMISH and QPCR respectively. The expression patterns provided the identification of the players and their order and place of appearance. The functional regulatory connections between the active regulatory genes were then obtained by a perturbation analysis, where every regulatory gene in the network was perturbed, and the effect on every other gene in the network was assessed by QPCR and whole mount insitu hybridization (WMISH) [6]. For key regulatory genes the perturbation analysis was followed by *cis*-regulatory analysis, the precise identification and characterization of the *cis*-acting genomic sequences regulating the transcription of a gene [5]. *cis*-regulatory analysis reveals which regulatory connection are direct and what is the logic function that a *cis*-regulatory module executes on its direct inputs. The network topology, the response functions of the *cis*-regulatory modules and the spatio-temporal expression patterns of the network genes were then incorporated into a comprehensive model. This linear diagram is of course a simplification of the actual research process which goes back and forth in order to improve our understanding in the light of new findings.

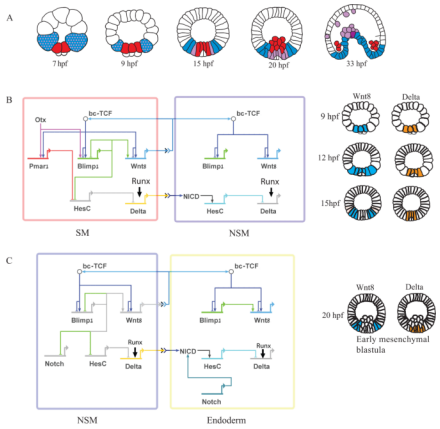

Figure 2.

Genomic code of the dynamic expression pattern of signaling pathways. (A) Schematic diagrams of the sea urchin (*Strongylocentrotus purpuratus*) embryo development. Red cells at 7 hpf are the large micromeres, from their descendants the skeleton forms. The blue-purple tier of cells at 7 h is the macromeres. Their descendants form mesoderm (purple) and endoderm (blue) cells. (B) Right, the circuit components active in the SMs and NSMs until 8th cleavage. Left, diagrams of the expression pattern of the Wnt8 and Delta ligands at these stages. (C) Right, the circuit components active in the NSM and endoderm at early mesenchyme blastula. Left, diagrams of the Wnt8 and Delta expression pattern at this time.

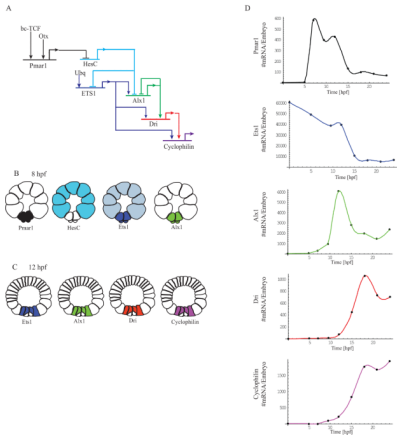

Figure 3.

The skeletonic lineage specification and differentiation. (A) Partial gene regulatory network of some of the skeletonic genes, including the double negative gate of Pmar1 and HesC and a cascade of feedforward loops constructed by the transcription factors Ets1, Alx1 and Dri, and the differentiation gene *cyclophilin*. (B) Expression pattern of Pmar1, HesC, Ets1 and Alx1 at 8 hpf. (C) Expression pattern of Ets1, Alx1, Dri and Cyclophilin at 12 hpf. D. Time courses of the mRNA level of thegenes *pmar1*, *ets1*, *alx1*, *dri* and *cyclophilin* measured by QPCR (Data obtained from ref. [7], Supplementary Material).

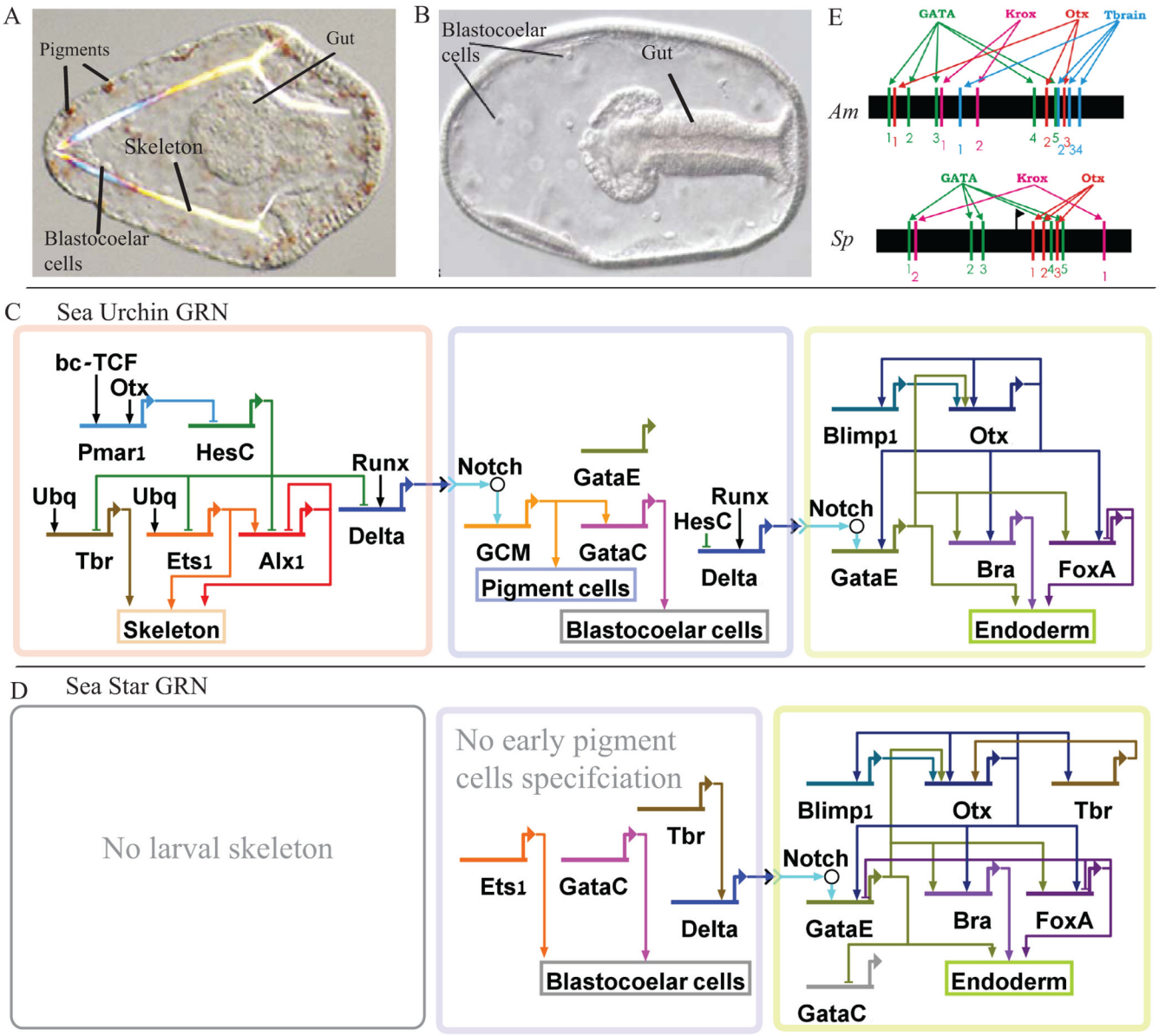

Figure 4.

GRN evolution underlies the evolution of body plans. (A). Sea urchin larva at 72 hpf (*S. purpuratus*, reprinted from ref. [31]). (B) Sea star larva at 70 hpf (*A. miniata*, reprinted from ref. [40]). (C) Endomesoderm circuits controlling the specification of the sea urchin skeleton, pigment cells, blastocoelar cells and endoderm. (D) Endomesoderm circuits controlling the specification of sea star blastocoelar cells and endoderm. (E) The mechanism of network rewiring – comparing the *cis*-regulatory modules of the *otx* gene in sea star and sea urchin reveals the change and conservation of the genomic code (Reprinted from ref [16]).